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Keywords

Itk and Btk, regulatory spine, kinase activation, gatekeeper residue, phosphorylation

Disciplines

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Identification of an allosteric signaling network within Tec family kinases

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Abstract

The Tec family kinases are tyrosine kinases that function primarily in hematopoietic cells. The catalytic activity of the Tec kinases is positively influenced by the regulatory domains outside of the kinase domain. The current lack of a full-length Tec kinase structure leaves a void in our understanding of how these positive regulatory signals are transmitted to the kinase domain. Recently, a conserved structure within kinases, the 'regulatory spine', has been identified that assembles and disassembles as a kinase switches between its active and inactive states. Here we define the residues that comprise the regulatory spine within Tec kinases. Compared to previously characterized systems, the Tec kinases contain an extended regulatory spine that includes a conserved methionine within the C-helix and a conserved tryptophan within the SH2-kinase linker of Tec kinases. This extended regulatory spine forms a conduit for transmitting the presence of the regulatory domains of Tec kinases to the catalytic domain. We further show that mutation of the gatekeeper residue at the edge of the regulatory spine stabilizes the regulatory spine resulting in a constitutively active kinase domain. Importantly, the regulatory spine is preassembled in this gatekeeper mutant rendering phosphorylation on the activation loop unnecessary for its activity. Moreover, we show that the disruption of the conserved electrostatic interaction between Btk R544 on the activation loop and Btk E445 on the C-helix also aids in the assembly of the regulatory spine. Thus, the extended regulatory spine is a key structure that is critical for maintaining the activity of Tec kinases.

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INTRODUCTION

Protein kinases catalyze the transfer of a phosphate group from ATP to a hydroxyl containing amino acid side chain; either Ser/Thr for serine-threonine kinases or Tyr for tyrosine kinases[1]. The activity of protein kinases is exquisitely regulated within the cell[1;2;3;4]. The switch from the catalytically inactive state of a kinase to the active state is often accompanied by the phosphorylation of a key residue within the activation loop, a large flexible loop that lies between the two lobes of the kinase domain structure[1]. Phosphorylation of the activation loop residue can trigger concerted movements in other

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mobile elements within the kinase domain such as the C-helix and the DFG motif that brings about the assembly of the catalytically critical residues in the kinase active state[1]. The active state conformations associated with different kinases are nearly identical across the many kinases for which high resolution structures have been solved[2;3;5;6;7]. It is not surprising that the active states of distinct kinases are very similar since the phospho-transfer chemistry carried out by different kinases is the same. In contrast, it is becoming clear that the structural features associated with the inactive state of various kinases differ widely.

The search for features that are conserved within the structures of active kinases has recently led to the identification of a structure termed the ‘regulatory spine’[8;9]. The regulatory spine was first identified by Local Spatial Patterns alignment analysis using a set of serine-threonine and tyrosine kinase structures that included PKA as a model kinase[8;9]. The regulatory spine defines a stretch of amino acid residues that is assembled only in the active state of kinases. Unlike consensus sequences that consist of a continuous stretch of amino acids in the primary structure, the regulatory spine consists of disparate residues that span the N- and C-terminal lobes of the kinase domain. Assembly of the regulatory spine has been proposed to be a crucial step in the activation of protein kinases and this structure is disrupted in structures of kinases in the inactive state. Moreover, the regulatory spine has been proposed as the mechanism by which allosteric effects can be propagated to the kinase active site[8;9].

The Tec kinases are immunologically related tyrosine kinases which consist of five mammalian members: Itk, Btk, Tec, Txk and Bmx[10;11]. Tec kinases share similar domain architecture as Src, Abl and Csk family of kinases, in that they have the SH3-SH2-kinase domain cassette[10]. Despite domain similarities, we and others have shown that the regulation of Tec kinases is distinct from Src and Abl kinases[10;12;13;14]. Unlike the Src and Abl kinases, the N-terminal regulatory domains of Tec kinases positively regulate the activity of the kinase domain[14]. While the isolated kinase domains of Src and Abl are active, the isolated kinase domains of the Tec family exhibit poor catalytic activity[13;14;15]. Regulation of the Tec kinases is in fact more similar to the Csk family of enzymes, whose N-terminal regulatory domains are essential for the catalytic activity of the kinase domain[16]. Moreover, while the structures of full-length Src and Csk kinases are available, the structure of a full-length Tec kinase remains elusive[2;17;18]. This leaves a significant gap in our mechanistic understanding of the regulation of the Tec family kinases.

In this manuscript, we define the regulatory spine within the Tec family kinases and show that mutation of the spine residues leads to different effects in the context of the isolated kinase domain verses full-length Itk. Moreover, we show that the assembly of a stable regulatory spine within members of the Tec kinase family is critically dependent on the presence of the SH2-kinase linker region. The residues that comprise the regulatory spine within PKA are insufficient to promote the assembly of a stable regulatory spine within the Tec kinases. We extend the Tec regulatory spine to include a conserved methionine within the C-helix and a conserved tryptophan residue within the SH2-kinase linker of Tec kinases. Together with the spine residues originally identified within the PKA kinase domain, the conserved methionine and tryptophan residues form a continuous structure that links the kinase active site to the N-terminal regulatory domains of Tec kinases. Furthermore, we show that the stabilization of the regulatory spine by the gatekeeper threonine to methionine mutation eliminates the need for phosphorylation on the activation loop for Btk kinase activity. Thus, the extended regulatory spine is a structure that is critical for the regulation of Tec kinases and if stabilized appropriately, is sufficient for activation in the absence of activation loop tyrosine phosphorylation and in the absence of the non-catalytic Tec regulatory domains.

RESULTS

The regulatory spine controls Tec kinase activity

The regulatory spine within PKA consists of five residues: L95, L106, Y164, F185, and D220[9]. These residues are spread throughout the primary sequence of the PKA kinase domain: L95 is located on the C-helix, L106 is on the N-terminus of the β4 strand, Y164 is part of the 'HRD' motif, F185 is from the 'DFG' motif and D220 is on the F-helix within the C-terminal lobe[9]. Alignment of the structure of the Btk and Itk kinase domains with that of PKA shows that the corresponding regulatory spine residues within the Btk and Itk kinase domains should consist of: Btk M449, L460, H519, F540 and D579 and Itk M409, L420, H479, F500 and D539 respectively (Fig. 1a).

To test the role of the predicted spine residues in the Tec kinases, we wished to take advantage of the rapid bacterial expression and purification system that has been developed for the isolated kinase domains of Itk and Btk[19]. One issue that arises, however, is the fact that the isolated kinase domains of the Tec kinases exhibit poor catalytic activity and so the expected loss of function mutations in the regulatory spine would be difficult to characterize given the already low activity of the wild type kinase domains. In order to take advantage of the ease of the bacterial expression system, we needed an isolated Btk and Itk kinase domain with higher catalytic activity. It has been demonstrated previously that introduction of a bulky hydrophobic residue such as isoleucine or methionine at the gatekeeper position activates multiple kinases[20]. Indeed, we find that the Itk F434M and Btk T474M isolated kinase domains are more active when compared to wild-type isolated kinase domain (manuscript in preparation). We therefore probed the importance of the predicted regulatory spine residues in Itk and Btk by mutating them individually to alanine in the context of the Itk F434M and Btk T474M isolated kinase domain mutants. Activity measurements are carried out by monitoring the phosphorylation levels of a peptide substrate in a radioactive assay or by detecting the level of autophosphorylation on the activation loop tyrosine (Y511 in Itk and Y551 in Btk) by western immunoblotting. While phosphorylation of the activation loop tyrosine is achieved by the activity of the Src family kinases Lck and Lyn respectively *in vivo*, under *in vitro* conditions Tec kinases autophosphorylate on the activation loop tyrosine[21;22;23].

Disruption of the regulatory spine residues by mutation to alanine is predicted to disrupt kinase activity. Indeed, mutation of Btk M449, H519, F540 and D579 and Itk M409, H479, F500 and D539 to alanine within the isolated kinase domain of Itk F434M and Btk T474M leads to decreased phosphorylation on the activation loop tyrosine and drastically reduces the catalytic activity (Fig. 1b, c, d and e). Itk kinase residues such as M409, F500 and H479 are highly conserved with other kinases with well-defined roles in catalysis[8]. Itk M409 is involved in binding the substrate ATP[12]. Itk F500 is part of the 'DFG' motif at the start of the activation loop segment and is responsible for stabilizing the conformation of the preceding aspartate, and the C-helix[8]. Itk H479 is part of the 'HRD' motif where it serves as a scaffold for D499 and F500[8]. It is therefore not surprising that mutation at these sites leads to a loss in activity. However, Itk D539 is not part of any previously characterized regulatory motif and therefore the drastic reduction in Itk activity upon mutation of this residue to alanine highlights the importance of the regulatory spine in regulating Itk activity. Moreover, since the regulatory spine residues are conserved within the Tec kinase family, this structure would be predicted to be critical for regulating the activity of all Tec kinase family members.

Unexpectedly, mutation of Itk L420 and Btk L460 to alanine, which based on the previous PKA work is predicted to be part of the regulatory spine, failed to inactivate the isolated kinase domains of Itk F434M or Btk T474M (Fig. 1b, c, d and e). In fact, the isolated kinase domains of both Itk L420A/F434M and Btk L460A/T474M double mutants showed an increase in activity when compared to the isolated kinase domain of Itk F434M and Btk T474M, respectively. To ensure that the activating effect of the Leu to Ala mutation was not an artifact of working with the activated (gatekeeper mutant) isolated kinase domains of Itk and Btk, the regulatory spine residues were mutated to alanine in the context of wild-type isolated kinase domains of Itk and Btk. Since the catalytic activity of the isolated kinase domain of Tec kinases is generally poor, the detection of activation loop phosphorylation levels by western immunoblotting is difficult and for Itk, in particular, phosphorylation Y511 is below the detection limit by western immunoblotting. Hence, the activity of the wild-type and regulatory spine mutants of the isolated kinase domains of Itk and Btk are monitored by their ability to phosphorylate a peptide substrate in the radioactive assay.

The results of the regulatory spine mutations in the context of the wild type kinase domains mirror the results for the activating gatekeeper mutants described above. Mutation of Btk M449, H519, F540 and D579 to alanine within the isolated wild type kinase domain of Btk leads to decreased catalytic activity (Fig. 1f). Circular dichroism spectra of the Btk mutants overlay well with that of wild-type Btk showing that the decreased activity of the Btk mutants are not due to unfolding of the kinase (Supp. Fig. 1). Since the activity of the isolated Itk wild-type kinase domain is not significantly above background levels, the resulting activity of the Itk spine mutants: Itk M409A, H479A, F500A and D539A can not be interpreted except to say activity is no greater than wild type (Fig. 1g). However, consistent with our earlier results with the gatekeeper mutant, the Itk L420A and Btk L460A mutants are both more active than wild-type isolated kinase domain of Itk and Btk, respectively (Fig. 1f and g). Interestingly, previous studies on the regulatory spine within the Abl kinase have shown that mutation of Abl L320 to glycine (corresponding to Itk L420 and Btk L460), leads to only a slight decrease in kinase activity of full length Abl[20]. This is also consistent with modeling studies of Abl that have shown that Abl L320 has a modest impact on regulatory spine formation[20]. Thus, Itk L420 and Btk L460 do not play a major role in the assembly of the regulatory spine within the context of the *isolated kinase domain* of Itk and Btk.

The regulatory spine is not assembled in the structure of phosphorylated Itk kinase domain

Activation of Tec kinases requires the phosphorylation of a conserved tyrosine within the activation loop[12;21;23]. Separate high-resolution structures of the Btk kinase domain with Y551 either phosphorylated (active) or unphosphorylated (inactive) have recently been reported[24]. A comparison of the inactive and active states of Btk kinase domain shows clear differences in the region of the regulatory spine (Fig. 2a). The regulatory spine residues assemble into a linear arrangement in the structure of the active Btk kinase, while interactions between the regulatory spine residues (in particular L460, M449 and F540) are disrupted in the structure of inactive Btk kinase. The structural differences in this region between active and inactive Btk are consistent with the role of the regulatory spine as defined previously for PKA (Fig. 2a).

Crystal structures of the isolated kinase domain of Itk that is either unphosphorylated or phosphorylated on Y511 in the activation loop are also available[12]. Surprisingly, the available Itk kinase domain structures overlay quite well, with little or no conformational differences between them regardless of the phosphorylation state of the activation loop. Inspection of the Itk kinase domain structures shows that the regulatory spine residues in the Itk kinase domain structures adopt a disrupted configuration similar to the inactive Btk kinase domain (Fig. 2b). An additional hallmark of an active kinase domain structure is the formation of a crucial ion pair between a conserved lysine positioned within the β3 strand of the kinase domain and a conserved glutamate on the C-helix[1]. The distance between the corresponding residues (Itk K390 and E405) within the structures of both phosphorylated and unphosphorylated Itk kinase domain is on the order of 8.0 Å which is significantly greater than the 3 to 4 Å distance that is observed in the structures of other active kinases (Fig. 2b)[12]. Indeed, the distance between the same conserved ion pair within Btk, (Btk K430 and E445) changes from 14.6 to 3.8 Å upon activation of the kinase (Fig. 2a). These observations suggest that both the phosphorylated and unphosphorylated Itk kinase domains adopt a conformation consistent with the 'inactive' enzyme. The inability of the Itk kinase domain to fully assemble into an active conformation, despite being phosphorylated on Y511 in the Itk activation loop, points to additional requirements for activation.

A notable difference between the Itk and Btk crystal structures is that the construct used for the crystallization of the Btk kinase domain included the SH2-kinase linker, while that of the Itk kinase did not (Fig. 2a & b)[12; 24]. We hypothesize that the absence of the SH2-kinase linker within the Itk construct used for crystallography might prevent the assembly of a stable regulatory spine in the kinase domain despite phosphorylation on the activation loop Y511.

Extension of the Tec kinase regulatory spine

We have previously shown that the SH2-kinase linker, the 17 amino acids between the SH2 domain and the kinase domain, is critical for the activity of both Btk and Itk, exerting a positive effect on the catalytic function of the kinase domain[14]. More specifically, a conserved tryprophan (Itk W355, Btk W395) in the SH2-kinase linker, as well as a methionine residue in the C helix (Itk M410, Btk M450) are crucial residues in the Tec family kinase regulatory apparatus (Fig. 3a)[14]. Like the regulatory spine mutants described in Figure 1, point mutations of Itk W355, Btk W395 or Itk M410 to alanine all result in a significant drop in catalytic activity (Fig. 3b). Examination of the crystal structures of Btk shows that Btk W395 and Btk M450 are located at the 'top' of the regulatory spine and, in the active Btk structure, serve to extend the hydrophobic packing of the spine residues well into the N-terminus of the kinase domain (Fig. 3c & d). In the inactive Btk structure, repositioning of the W395 and M450 side chains disrupts the extended regulatory spine (Fig. 3c & d) in much the same manner that repositioning of M449 in the inactive Btk structure disrupts the core of the spine (Fig. 2a). The active and inactive Btk structures consist of the same amino acids (residues 382–659) and differ with respect to the activation loop tyrosine (Y551). In the active structure the activation loop tyrosine is mutated to glutamate (Y551E) to mimic phosphorylated Btk, and for the inactive structure wild type Btk in the unphosphorylated form was used for crystallization[24].

Compared to the regulatory spine that has been defined for PKA[9], the Tec kinase family requires at least two additional residues (Itk W355 & M410) to fully assemble the regulatory spine. The tryptophan residue is located in the SH2-kinase linker region providing an explanation for the positive regulatory role of this non-catalytic region[14]. The available Itk kinase domain structures are entirely consistent with this finding; without the contribution of the SH2-linker region, the regulatory spine of Itk does not assemble into the active conformation. As a result, the structures of the phosphorylated and unphosphorlated Itk kinase domain fragments are very similar to each other and neither resembles an active kinase. Future structures of Tec kinases with and without the SH2-kinase linker in various activation (phosphorylation) states will be required to fully probe this hypothesis.

Itk L420 and Btk L460 are essential for the formation of the extended regulatory spine within full-length Tec kinases

The conserved tryptophan in the SH2-kinase linker is critical for the activity of full-length Tec kinases[14]. Upon activation, the tryptophan side-chain forms part of a hydrophobic pocket that is lined by the conserved methionine on the C-helix and leucine on the β4 strand (Itk M410, L420 and Btk M450, L460) (Fig. 3d). Although Itk L420 and Btk L460 were shown to *not* be a part of the regulatory spine in the context of the *isolated kinase domain* (Fig. 1), the structure of the Btk kinase domain (which contains the SH2-kinase linker region) suggests that this leucine would be critical for the formation of the extended regulatory spine in the context of the *full-length* kinase. We therefore tested the role of this leucine in the context of full-length Itk.

As shown in Figure 3e, mutation of Itk L420 to alanine within the context of full-length Itk disrupted the activity of Itk. Consistent with our earlier results, mutation of Itk M409, F500 and D539 to alanine within full length Itk also inactivated the kinase. Problems with cloning prevented us from testing the activity of the full-length Itk H479A mutant. Together, Itk M409, L420, H479, F500, D539, M410 and W355 form a conserved allosteric signaling network that is absolutely required for the activity of Itk. Based on sequence conservation, the extended regulatory spine defined here likely controls the activity of the entire Tec kinase family.

Phosphorylation on the activation loop tyrosine is not required for the activity of the Btk T474M mutant

It is well established that activation loop phosphorylation is required for activation of tyrosine kinases[1;8] and specifically for the Tec kinases, phosphorylation on the activation loop tyrosine results in at least a ten-fold increase in activity[21;25]. Structures of active and inactive kinase domains from a number of different kinases demonstrate a conserved electrostatic network that switches between the active and inactive conformations (Fig. 4a) [1;8]. In the inactive state of Btk, the conserved glutamate (Btk E445) on the C-helix (of the Lys-Glu ion pair) is associated with a conserved arginine (Btk R544) on the activation loop[24;26]. Activation of Btk by phosphorylation on Btk Y551 (the activation loop tyrosine) leads to a specific interaction between pY551 and R544, and a concomitant loss of the association between the R544 and E445[24;26]. Btk E445 on the C-helix then swings toward the kinase active site and associates with the conserved lysine side chain (Btk K430) to bring about the assembly of the active state of the kinase[24; 26]. The conformational adjustment of the C-helix brings M449 and M450 in line with the other residues of the regulatory spine (Fig. 4a). Thus, phosphorylation on the activation loop tyrosine is an initiating step in the process of regulatory spine assembly.

Since mutation of the gatekeeper residue to methioine appears to stabilize the regulatory spine within Btk, we next probed the requirement of activation loop phosphorylation in the context of the T474M gatekeeper mutation. The activity of the BtkT474M/Y551F double mutant was compared to that of the Btk T474M mutant by monitoring phosphorylation of an exogenous substrate. We find that mutation of the activation loop Btk Y551 to phenylalanine has no effect on the activity of the Btk T474M mutant (Fig. 4b & c). The Btk T474M/Y551F double mutant and the Btk T474M single mutant are both active, whereas the wild type Btk kinase requires activation loop phosphorylation; the Btk Y551F single mutant exhibits poor activity (Fig. 4b). These data are consistent with the idea that phosphorylation on the activation loop tyrosine initiates the assembly of the regulatory spine by disrupting the association between Btk E445 and Btk R544. Once pY551 competes with E445 for association with R544, the E445/K430 salt bridge is formed, bringing the C-helix (and importantly M449) into the regulatory spine structure. Under conditions that pre-organize the regulatory spine structure (as in the Btk T474M mutant), phosphorylation on the activation loop Y551 is no longer required to trigger the conformational changes that accompany Btk activation.

We further tested the importance of this switched electrostatic network by mutating Btk R544. We reasoned that mutation of Btk R544 will activate the kinase as inward movement of the C-helix is restrained by the E445:R544 interaction and loss of this electrostatic interaction should facilitate movement of the C-helix. Indeed, mutation of Btk R544 to serine in the context of wild-type isolated kinase domain of Btk leads to a two-fold increase in activity (Fig. 4d & e). In contrast, mutation of Btk R544 to serine in the context of Btk T474M isolated kinase domain does not further activate the kinase (Fig. 4d & e). These results again suggest that pre-assembly of the kinase regulatory spine (by mutation) can overcome the regulatory interactions that normally control kinase activity. Thus, for the wild-type kinase under physiological conditions, phosphorylation on the activation loop sets into motion a cascade of events: (1) formation of the pY551:R544 interaction with concomitant disruption of the Btk E445:R544 interaction, (2) inward movement of the C-helix and formation of the E445:K430 interaction and (3) assembly of the regulatory spine, that culminates in the formation of an active kinase (Fig. 4a).

DISCUSSION

The structures of the isolated kinase domain of Btk in both the active and inactive states have provided significant insight into Tec kinase activation[24;26]. However, the lack of a full-length structure of any Tec kinase leaves numerous unanswered questions regarding the regulation of Tec kinases. While the SH2 domain and the SH2-kinase linker region of Tec kinases have been shown to be required for the activity of Tec kinases[12;13;14], the mechanism by which these regulatory domains positively influence catalytic activity has not been clear. Here we identify an intramolecular allosteric signaling network that extends from the SH2-kinase linker into the kinase domain of Tec kinases. This intramolecular connectivity defines how the non-catalytic Tec regulatory domains, through the SH2-kinase linker region, impinge on the kinase domain and stabilize the regulatory spine. In the absence of the Tec regulatory domains, whether achieved by deletion or conformational changes within the full-length molecule, the regulatory spine is disrupted and kinase activity is inhibited.

Comparing our results to those previously published for PKA shows that the Tec kinases require an extended spine structure and it is this longer regulatory spine that couples kinase activity to regions outside of the kinase domain. Specifically, a conserved methionine in the C-helix and a conserved tryptophan in the SH2-linker swing in to ‘cap’ the spine in the active Btk structure and are critical for the activity of the Tec kinases. Mutation of these extended spine residues disables kinase activity even if the ‘core’ residues defined for PKA are intact.

Further support for the importance of the entire regulatory spine comes from examination of the genetic mutations in Btk that are associated with X-linked agammaglobulinemia (XLA) in humans, a disease that is characterized by impaired B cell development[27]. Several of the extended regulatory spine residues identified in this study are mutated in XLA patients[27]. In addition to nonsense mutations at Btk M449, M450 and W395, which cause premature truncation of the protein, there are also missense mutations at Btk M450, F450 and D579 (M450I, F450S and D579V). These mutations likely destabilize the regulatory spine and prevent Btk kinase activation.

Activation loop tyrosine phosphorylation has long been known to influence kinase activity. In addition to priming the kinase domain for the assembly of the regulatory spine, phosphorylation on the conserved Tyr of the activation loop is also thought to stabilize the substrate-binding site of the activation loop[1]. However, our work has shown that in the context of the activating gatekeeper Btk T474M mutation, loss of Y551 phosphorylation (by mutation to phenylalanine) has no affect on substrate phosphorylation. Pre-assembly of the regulatory spine (mutation of T474M) removes any requirement for activation loop phosphorylation. These results are consistent with earlier studies that focused on phosphorylation of Y551 in Btk[25]. That earlier work did show that phosphorylation on Btk Y551 significantly alters catalytic activity of Btk; k_{cat} of the Btk Y551F mutant is greatly diminished compared to wild-type Btk presumably due to loss of the trigger for regulatory spine assembly. However, the affinity for a peptide substrate is unaffected by Y551 phosphorylation; K_m of a peptide substrate for Btk Y551F mutant is the same as wild-type Btk. As well, an alternative direct substrate docking mechanism has been identified for the Tec kinases, in which an SH2 domain within the substrate docks onto the kinase domain outside of the active site and facilitates substrate phosphorylation[28;29;30]. It is therefore possible that the peptide substrate-binding region on the activation loop may only play a minor role in substrate recognition in Tec kinases and that activation loop phosphorylation plays one significant role: initiation of spine assembly by altering electrostatic interactions in and around the active site.

We have previously shown that the regulation of Tec family kinases is similar to that of Csk (C-terminal Src kinase)[14]. Unlike the Src and Abl family of kinases, the SH3 and SH2 regulatory domains of Csk and Tec kinases positively influence the catalytic activity of the kinase domain[14;16]. Like Itk and Btk, the isolated kinase domain of Csk has poor catalytic activity[16;31;32]. In order to define the elements within the Csk kinase domain that are responsible for the poor catalytic activity, a mutagenesis study was carried out in which Src:Csk kinase domain chimeras were created[33]. A Src:Csk chimeric kinase domain where the N-terminal lobe of Csk was replaced with the N-terminal lobe of Src was shown to exhibit activity comparable to that of full-length Csk[33]. Further mutagenesis of the N-terminal lobe, identified the C-helix and the β turn between the β4 and β5 strands (the ‘top’ of the regulatory spine) as key structural elements that are required for the activity of the isolated kinase domain of Csk[33].

There is remarkable correlation between the elements that are identified in the Csk mutagenesis study and the extended regulatory spine of Tec kinases identified in this study. Since the Csk and Tec family kinases are both positively regulated by domains outside of the kinase domain, it is not surprising that they would share the key determinants of catalytic activity. Indeed, as suggested in the original identification of the kinase regulatory spine[9], this feature likely plays a critical role in all active kinases. Our work confirms this notion for the Tec family kinases, and illustrates that extension of the regulatory spine by two residues couples this regulatory feature to allosteric events occurring outside of the kinase domain. Moreover, our data suggest that despite a high degree of sequence conservation, the five core regulatory spine residues do not assemble into the active conformation in the context of the isolated Itk or Btk kinase domains. The energetics of spine assembly seem to be tailored for each specific regulatory environment; the Tec kinase domains are maintained in their inactive state (spine assembly does not occur even when the activation loop is phosphorylated) until additional regions of the protein (in this case the SH2-linker) are present to drive complete spine assembly forward and fully activate the kinase.

MATERIALS and METHODS

Constructs

The baculoviral expression constructs for full-length Itk has been described previously[14]. The bacterial expression constructs for the Itk kinase domain has been described elsewhere[19]. The mouse wild-type Btk kinase domain (396-659) was PCR amplified and cloned into the pET 28b (Novagen) vector to create the His-tagged Btk kinase domain. All mutations were introduced by using the site directed mutagenesis (SDM) kit (Stratagene).

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All constructs were verified by sequencing at the Iowa State DNA synthesis and sequencing facility. Mouse numbering is used throughout for both the Itk and Btk sequences.

Protein expression and purification

Baculoviral constructs were expressed and purified from *Sf*9 cells as described previously[14]. The bacterial expression constructs for the His-tagged Itk, Btk kinase domains were expressed and purified from ArcticExpress cells (Stratagene) as described previously[19]. Briefly, the Itk or Btk kinase domains were expressed in ArcticExpress bacteria at 12°C for 23 hours. The cell pellets were re-resuspended in lysis buffer (0.5 mg/ml lyzozyme, 50 mM KH_2PO_4 pH 8.0, 150 mM NaCl, 20 mM imidazole) and stored overnight at −80°C. The cell pellets were thawed after the addition of 1 mM PMSF and 3000 Units DNase I (Sigma). The lysate was spun at 14K for 1 hour at 4°C. The supernatant was incubated with Nickel NTA resin (Qiagen). The resin was washed with wash buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 40 mM imidazole) and then eluted with elution buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 250 mM imidazole, 10% glycerol). The proteins were aliquoted, flash frozen with liquid nitrogen and stored at −80°C. All protein preparations were greater than 95% pure as assessed by Coomassie Blue staining of the gel.

Kinase assays and western blotting

In vitro kinase assays were performed by incubating either full-length or the isolated kinase domain of Itk or Btk in a kinase assay buffer (50 mM Hepes pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, 1 mM Pefabloc and 200 μM ATP) at RT for one hour. The samples were boiled, separated by SDS-PAGE and western blotted with the Anti Btk phosphoY551 antibody (BD Biosciences), or anti-His (Upstate) antibody as described previously[29]. Anti Btk pY551 is used throughout to detect both phosphorylation on Btk Y551 and Itk Y511. Kinetic parameters for the full-length wild-type and mutant Itk are derived using radioactive assays that have been described previously[14].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Itk	Interleukin-2 tyrosine kinase
Btk	Bruton's tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
PKA	Protein Kinase A
ATP	adenosine triphosphate

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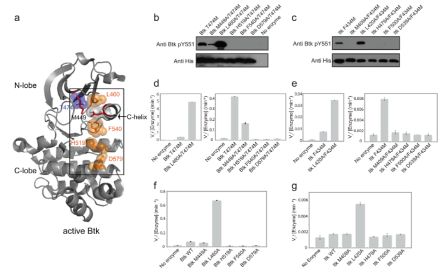

Figure 1. Identification of the regulatory spine in Tec kinases

(a) The regulatory spine is assembled in the active Btk kinase domain (PDB ID 3GEN). The predicted regulatory spine residues, D579, H519, F540, L460, are shown in orange (sticks plus space filling model) with M449 on the C-helix shown in grey. The gatekeeper residue, T474, is shown in blue. The conserved ion pair, K430 and E545, in the active Btk conformation are shown using red sticks but not labeled. The C-helix and the amino (N) and carboxy (C)-lobes of the kinase domain are labeled. All structures in this and other figures were generated using PyMOL[34]. (b, c) The activity of the Tec kinase mutants correlate with the level of phosphorylation on the activation loop of the kinase. The purified isolated kinase domains (residues 396-659 for Btk and residues 356-619 for Itk) were concentrated to 500 nM and incubated in a kinase assay buffer at RT for one hour, separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane, and probed with either an anti-Btk pY551 or anti-His antibody. The regulatory spine residues: Btk M449, L460, H519, F540 and D579 and Itk M409, L420, H479, F500 and D539 were separately mutated to Ala in the context of Btk T474M isolated kinase domain (b) or Itk F434M isolated kinase domain (c). (d, e, f & g) The regulatory spine residues were mutated to Ala in the context of either Btk T474M kinase domain (d) or wild-type Btk kinase domain (f), Itk F434M kinase domain (e) or wild-type Itk kinase domain (g). Activity (specifically initial velocity, V_i) is measured in an *in vitro* kinase activity using Peptide B as a substrate as described previously[14]. For (d) and (e) the data for the activating Leu mutation in Btk and Itk are shown in a separate panel for clarity.

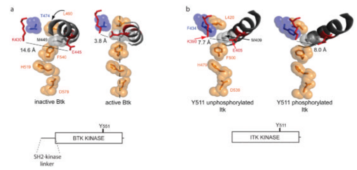

Figure 2. The regulatory spine is assembled only in the active state of kinases

Comparison of the regulatory spine of (a) inactive and active Btk structures (PDB IDs 3GEN and 3K54 respectively) and (b) Y511 unphosphorylated Itk (PDB ID 1SNU) and Y511 phosphorylated Itk (PDB ID 1SM2). As in Figure 1, the regulatory spine residues are orange the gatekeeper residue is shown in blue. The assembled regulatory spine in active Btk in indicated with a dotted line. The distances between the critical conserved ion pair (red sticks) that is a hallmark of kinase activation are indicated for each structure. A schematic of the constructs used for crystallization of the Btk and Itk kinase domains are indicated below the structures. The Btk construct includes the SH2-linker region preceding the kinase domain and extends to the C-terminus while the Itk construct was limited to the isolated kinase domain.

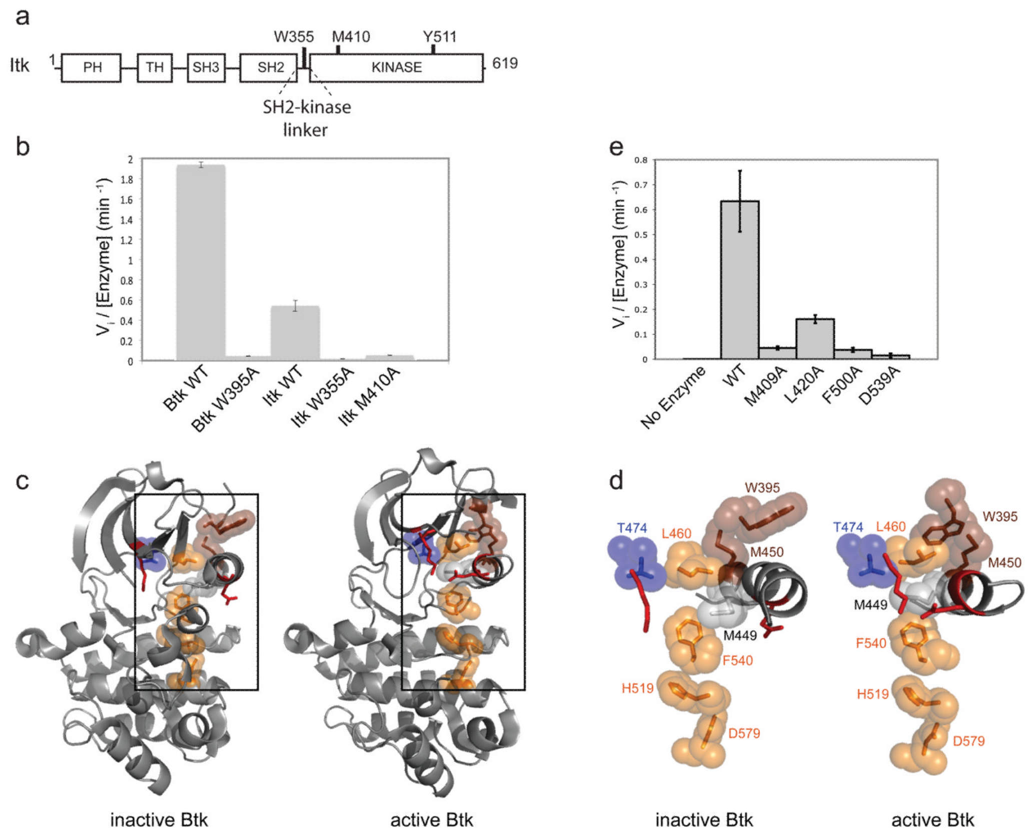

Figure 3. Extension of the regulatory spine within Tec kinases to include the SH2-kinase linker

(a) Schematic of the domain architecture of full length Itk with the SH2-kinase linker region and additional spine residues indicated. (b) Btk W395, Itk W355 and Itk M410 were mutated to alanine within full-length Btk or Itk respectively and tested for their *in vitro* kinase activity using Peptide B as a substrate. (c and d) The Btk M450 (M410 in Itk) and Btk W395 (W355 in Itk) form an extension of the regulatory spine to create an allosteric network connecting the SH2-linker region to the kinase domain. The crystal structures of inactive and active Btk are shown with the location of the E/K salt bridge (red sticks unlabeled), regulatory spine residues (D579, H519, F540, L460: orange, M449: grey), gatekeeper residue T474 (blue), and M450 on the C-helix and W395 in the SH2-kinase linker (brown). The boxed regions in (c) are enlarged in (d). (e) The regulatory spine residues: Itk M409, L420, F500 and D539 were mutated to alanine in the context of full-length Itk and tested for activity by monitoring Peptide B phosphorylation as in Figure 1.

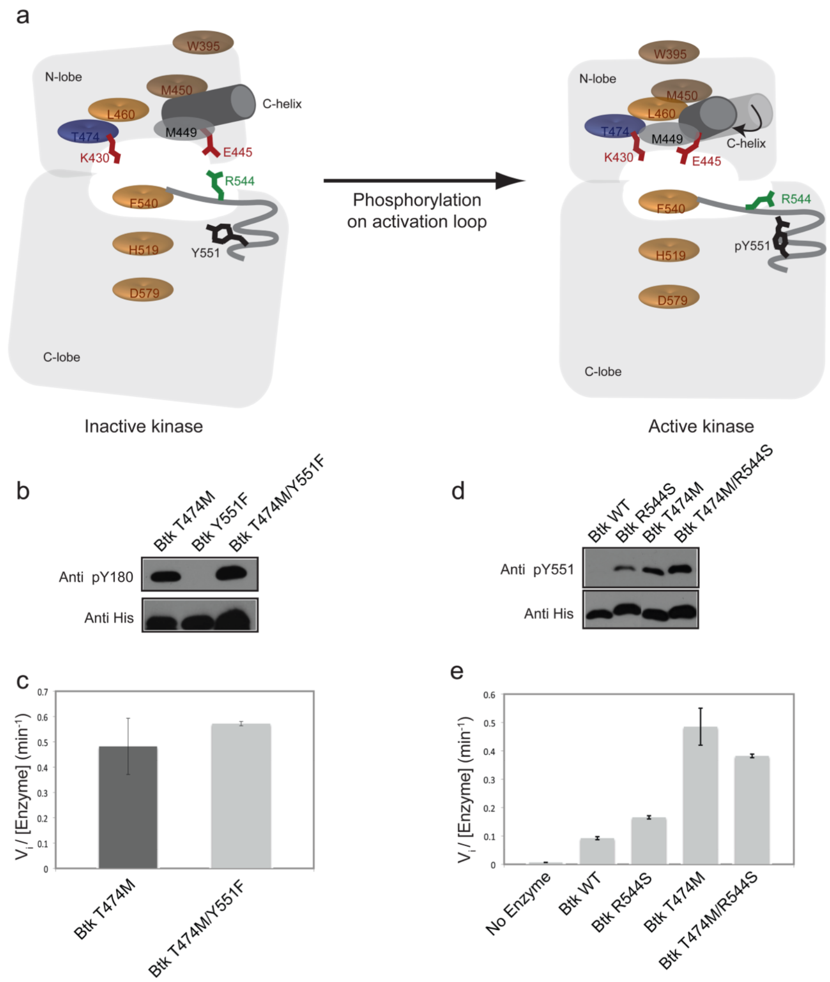

Figure 4. The regulatory spine is preassembled in the Btk T474M gatekeeper mutant

(a) Cartoon showing the key components of the allosteric network within Tec kinases. Tec kinases have an extended regulatory spine, which includes residues beyond the isolated kinase domain: specifically the conserved Trp in the SH2-kinase linker. The residues that make up the regulatory spine (orange disks) are not assembled in the inactive conformation of the kinase. Phosphorylation on the activation loop triggers the assembly of the regulatory spine. Phosphorylation on the activation loop engages Btk R544, which then releases Btk E445 on the C-helix to swing inwards and form the conserved salt bridge with Btk K430. (b) The Btk T474M mutant does not require phosphorylation on the activation loop for its activity. The Btk T474M, Btk Y551F or Btk T474M/Y551F isolated kinase domain were incubated with a substrate (the SH3SH2 domain of Itk) in a kinase assay buffer for one hour at RT. Phosphorylation on the substrate was monitored by western blotting with a Btk pY223 antibody which has been used previously to recognize phosphorylation on Itk Y180[29]. (c) The Btk T474M/Y551F isolated kinase domain mutant is as active as Btk T474M isolated kinase domain. Btk T474M or Btk T474M/Y551F were tested for their *in vitro* kinase activity using Peptide B as a substrate as described previously[14]. (d & e) Disruption of the conserved electrostatic network by mutation of Btk R544 to Ser activates wild type Btk. Btk R544 was mutated to Ser in the context of wild-type isolated kinase domain of Btk or Btk T474M isolated kinase domain and tested for its activity as in Figure 1. In (d) autophosphorylation on the activation loop tyrosine is monitored and in (e) Peptide B phosphorylation is measured. The use of serine instead of alanine in this case is based on the location of this mutation in the exposed activation loop. We expected that the activation loop could accommodate a hydrophilic side chain more readily than a hydrophobic side chain.